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(54) Title: APPLICATION OF LYOLUMINESCENCE FOR ANALYTICAL PURPOSES

(57) Abstract

A method, wherein the excitation of label molecules is achieved by a dissolution of a solid material in a solvent followed by exploitation of the resulting luminescence for chemical and biochemical analyses. In the said process intermediates responsible for the excitation of labels are either dissolution-released and/or produced from the solid or solvent during dissolution, or produced from coreactants added in the solvent. Either one or several different label compounds can be excited to excited state(s) which is/are deexcited by emission of ultraviolet, visible or infrared light providing the basis for reproducible analytical applications in bioaffinity assays such as in immunoassays and DNA-probing assays.

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APPLICATION OF LYOLUMINESCENCE FOR ANALYTICAL PURPOSES

Field of invention

The present invention relates to excitation of label substances in biochemical and chemical analyses by unstable intermediates produced by bringing solid material into solvated state enabling the label substances finally emit light in UV, visible or IR range. The said solids are preferably strongly reducing metals or substances irradiated with high-energy electromagnetic radiation.

Background of invention

Processes where light is generated during dissolution of a solid are called lyoluminescence (LL) (G. Reynolds, *Journal of Luminescence,* 54, 1992, 43.). Some materials produce intrinsic LL where the emission is based solely on the reactions of the components of the solid and the solvent, and some materials mainly produce extrinsic LL where the emitter is an added component, solvated or otherwise present in the solvent. This invention relates to chemical and biochemical analysis where lyoluminescence is utilised in generation of light from label substances.

Luminescent (fluorescent, phosphorescent or chemiluminescent) label substances are utilised in various forms of biochemical and chemical analysis. Luminescent molecules allow to label other substances (X), or (B) which has an affinity to (X). The amount of (X) can be determined using various well-known technique principles. This kind of label substances, organic compounds or metal chelates, can be excited by light, electrochemical or chemical reactions thus producing luminescence specific to the label substance (I. Hemmilä, "*Applications of Fluorescence in Immunoassays*", John Wiley & Sons, New York, 1991; E. Diamandis, *Clinical Biochemistry*, 23, 1990, 437., L. Faulkner and A. Bard, in "*Electroanalytical Chemistry* " Vol. 10., A. Bard, (Ed.), Marcel Dekker, New York, 1977, pp. 191-228). LL with poorly known generation mechanisms has been utilised for dosimetry of irradiation or detection of compounds which

themselves are luminescent. For example, radioactive irradiation generates trapped electrons and holes in alkali halide crystals the amount of trapped charges being directly proportional on the irradiation dose. The dose can be quantitated by dissolving the irradiated crystals into a proper solution and by measuring the LL intensity during dissolution of the solid (G. Reynolds, *Journal of Luminescence,* 54, 1992, 43.).

It is known on the basis of studies in dosimetry that some metal ions or organic compounds can be excited in aqueous solution in LL processes induced by dissolution of irradiated solids (G. Reynolds, *Journal of Luminescence,* 54, 1992, 43.). However, the direct detection of rarely existing metal ions or complicated organic luminophores has no practical meaning in chemical analysis. In addition, in earlier studies bell-shaped calibration curves (two concentrations in a narrow concentration range yield the same response value) with very limited dynamic range were obtained (Figures 24 and 25, in G. Reynolds, *Journal of Luminescence,* 54, 1992, 43). This kind of undesired calibration curves and a very limited working range have not allowed the use of LL in practical analysis.

Bell-shaped calibration curves can be avoided with the techniques of the present invention and linear calibration curves on log-log coordinates extending over several orders of magnitude of concentration can be obtained. The procedures are generally inverse to those used in dosimetry, where the irradiation product has been added into a solution. With the present methods the source of chemical energy in LL processes is a solid, either a suitable metal or a solid irradiated with an exact dose of radiation, which solid is dissolved by a convective injection of a liquid sample on the solid, carrying the labelled substances, into the cell in a highly reproducible manner which allows the quantitation of the labelled substance and a certain analyte by measurement of the LL intensity. This invention relates to those cases where the analyte itself is not luminescent, but the amount of analyte is quantitated using LL-producing labels (L). Labels (L) are normally covalently bound either to analyte (X) or to a substance (B) which selectively binds with (X), such as antibodies or receptors. The amount of analyte can be determined either with a competitive reaction with (X) and labelled (X), (X-L), or by allowing labelled (B), (B-L), to specifically react

with (X) when a complex (X-B-L) is formed. In all cases, the finally measured parameter is the amount of label which is proportional to the amount of analyte.

Typically, the analyte (X) is a biomolecule which is quantitated by a binding with another biomolecule which has high specific affinity to (X). The analyte can be, for instance, a cell, haptene, nucleic acid, gene. All processes are possible in which the bound analyte (X-L, X-B-L or a corresponding biocomplex) can be separated by free label, (X-L) or (B-L). It is essential that the amount of analyte yields a direct or inverse proportionality of LL intensity measured after separation of the free label. The quantitation of analytes suitable for the present methods have considerable economical importance in biochemical, clinical, environmental and agricultural analysis. In conclusion, the present invention makes a considerable improvement over the prior art for a certain branch of methods in bioaffinity assays.

In an analysis based on binding properties of biological molecules, such as in an immunoassay, the analyte (X) can be selectively bound from a mixture of molecules on the surface of a solid support precoated with biomolecules, such as antibodies, and the amount of (X) can be quantitated with the aid of another biomolecule which also has a specific affinity against the analyte and is labelled with a suitable marker. The marker can be a radioactive isotope, an enzyme, a chromophore-containing moiety, or a luminophore, etc. which is bound to a biomolecule. Alternatively, a purified (X) can be labelled with a marker and utilised in determination of (X) by well-known competitive assay procedures. In addition to immunoassays, many DNA and RNA assays are based on analogous principles. Also other types of chemical and biochemical analyses can be performed using analogous procedures. Various methods and strategies in bioaffinity assays are described in the literature (*The Immunoassay Handbook*, Edited by David Wild, Stockton Press Ltd., New York, 1994, pp. 1-618).

Two main development branches in clinical, biochemical and environmental analyses are the use of well-equipped central laboratories and an analysis carried out outside the laboratories in nature, at doctors offices or at home of the patients. Both of these

branches have their own benefits, but the latter is only in the beginning of its development. Especially, rapid diagnostics and Point-of-Care markets have an enormously growing importance of the present day.

The main aim of the present invention is to fulfil the needs of the latter type of analyses. The invention can be used, for example, to monitoring the health of a patient at home, rapid diagnosis at physicians office, or in agricultural or environmental analysis in the field circumstances. In all these cases, fresh samples and rapid and easy determinations are important. However, the measurement instrument cannot be expensive and the assay procedure must be so safe and easy that untrained persons can carry out the analysis and still obtain reproducible results. On the other hand, because the number of analysis per day is limited, the price of the reagents can be considerably higher than in cases of an analysis carried out in central laboratories. In this area, optimal analysis method is based on a carefully developed assay kit, that can be used by non-experts. After addition of the sample the analysis result should be obtained easily and rapidly with a simple procedure.

Radioactive labels are not favourable in the applications directed tom unprofessional personnel due to the specific regulations. Simple photometers and the methods based on light absorption are generally too insensitive for many practical applications in this field of analysis. In addition, the need of optical components often makes spectrophotometers too expensive to be applied in the analyses. For special purposes, there are commercially available inexpensive portable luminometers which are capable of detecting very low light intensities and have photomultiplier or photodiode as a light detector. These luminometers are normally used in connection with biochemical light generation with the enzyme luciferase. In principle, luciferase can be used as a label, but then luciferase reaction must be coupled with formation or decomposition reactions of ATP/NADH, which makes the procedure difficult to carry out and subject to experimental errors due to very easily decomposed reagents. Thus, the storability of the kits is poor. Methods based on fluorescence and phosphorescence are sensitive, but the instrumentation is much more expensive than in the present methodology.

Detailed description of the invention

The aims of this invention are simple methods and apparatus for detection of label substances that can be utilised in bioaffinity assays, such as in immunoassays and DNA-probing assays, which are carried out by non-experts at home, at physicians offices, at farms or in the nature for environmental control.

The aims of the present invention can be reached by using luminescent labels under carefully chosen conditions so that the labels are excited by chemical reactions according to this invention. The generated light is measured with a very simple luminometer, or with an apparatus that has no electrical components with the aid of light-sensitive film according the claims 1-4 of this invention. The main advantages of utilisation of lyoluminescence are that expensive light sources, optics, potentiostats, electrode materials, dangerous substances to environment or to users are not required. The analysis can be carried out with a low-cost apparatus which is simple to use, and in some cases, an apparatus containing electronic components is not required at all. The methods themselves must be so well-developed that the use of apparatus is simple and sufficiently reproducible.

The basis of the present method is storing energy in solid substances and releasing the energy during dissolution of the solid as chemical energy which is partially used to excite label substances. The amount of label substance which is proportional to the amount of the analyte in the sample is excited by redox reactions produced by the dissolution-released radicals or by an energy transfer from primary excited species. Also some metals, such as aluminium or magnesium, can produce extremely reactive intermediates during their dissolution which intermediates can efficiently produce extrinsic lyoluminescence from label substances. Some of the mechanisms involved are described below.

X- or γ-ray irradiation induces storage of energy in the form of trapped electrons and holes in the irradiated solid, such as in an alkalihalide (reaction 1). However, irradiation

product which contains simultaneously trapped electrons and holes are not applicable in a real-world analysis because the product is sensitive to temperature and room light due to the easily occurring recombination of the trapped electrons and holes. These problems can be avoided when other coloration methods of alkalihalides are used, or irradiation methods producing only isomerisation or breakage of bonds in the solid are used. Energy can be stored in the solid reagents by additive coloration (reactions 2a-2b) or electrolytic coloration (reactions 3a-3b) (J. Schulman and W. Compton, "*Color Centers in Solids*", Pergamon Press, New York, 1962). An electron trapped in an halide ion vacancy is called as F-centre and a V-centre is a hole analogue of an F-centre.

$$\text{(1)}\quad \text{alkalihalide} \xrightarrow{h\nu > 10\ eV} F_{centre} + V_{centre}$$

$$\text{(2a)}\quad \text{alkalihalides} \xrightarrow[\text{high pressure and temperature}]{\text{alkali metal vapour under}} F_{centres}$$

$$\text{(2b)}\quad \text{alkalihalides} \xrightarrow[\text{high pressure and temperature}]{\text{halogen gas under}} V_{centres}$$

$$\text{(3a)}\quad \text{alkalihalides} \xrightarrow[\text{high temperature}]{\text{pointed cathode under}} F_{centres}$$

$$\text{(3b)}\quad \text{alkalihalides} \xrightarrow[\text{high temperature}]{\text{pointed anode under}} V_{centres}$$

A storage of energy can also be based on the breakage of bonds during irradiation, such as a production of solid solution of sulfate radical in potassium peroxodisulfate and phosphate radical in potassium peroxophosphate during UV-irradiation (reactions 4 and 5) or on an isomerisation (reaction 6).

(4) $S_2O_8^{2-} \xrightarrow{h\nu > 4eV} 2\ SO_4^{\cdot -}$

(5) $P_2O_8^{2-} \xrightarrow{h\nu > 4eV} 2\ PO_4^{\cdot 2-}$

(6) $KNO_3 \xrightarrow{h\nu > 4.8\ eV}$ potassium peroxonitrite

Energy stored by reactions (1) - (6) can be released by dissolving the solids in a solvent. Then radicals induced by the dissolution of the solid can produce luminescence by redox mechanisms which is, in some cases, followed by energy transfer from the intrinsic emission centres to the label substances (reactions 7a - 7h). In reactions (7c-7e), (A) denotes a label molecule that is capable of being excited by redox reactions. This label molecule can be a purely organic luminophore such as, a derivative of isoluminol or fluorescein) or a metal chelate (such as Ru(II)-polypyridine chelates or Tb(III)-chelates containing an aromatic moiety).

(7a) $F_{centre} \xrightarrow{H_2O} e_{aq}^-$ (hydrated electron)

(7b) $V_{centre} \xrightarrow{H_2O} X\cdot$ ($X\cdot = F\cdot$, $Cl\cdot$ or $Br\cdot$)

(7c) $A + e_{aq}^-$ (or $+ X\cdot$) $\rightarrow A_{red}$ (or $A_{ox} + X^-$)

(7d) A_{red} (or A_{ox}) $+ X\cdot$ (or e_{aq}^-) $\rightarrow A^*$

(7e) $A^* \rightarrow A + h\nu$ (light)

In some cases, the excitation of label molecules can partially or exclusively be based on the energy transfer from the primary excited species (reactions 7f-7h).

(7f) $e_{aq}^- + X\cdot \rightarrow (X^-)^*$

(7g) $(X^-)^* + A \rightarrow X^- + A^*$

(7h) $A^* \rightarrow A + h\nu$ (light)

If the solvent itself or its solutes can act as energetically suitable oxidant or reductant in the LL system, extrinsic LL can be generated by dissolution of a solid that produces only reducing or oxidising species in its dissolution process. For example, dissolution of additively coloured alkalihalides into a solution that contains suitable concentration of peroxodisulfate ions produces simultaneously strongly reducing and oxidising conditions, which allows to bring 6.3 eV of chemical energy, at maximum, in the excitation reaction pathways (reactions 8a-8b).

$$F_{centre} \xrightarrow{H_2O} e_{aq}^-, \quad E^o(e_{aq}^-) = -2.9 \text{ V vs. SHE}$$

(8a)

(8b) $e_{aq}^- + S_2O_8^{2-} \rightarrow SO_4^{-\cdot} + SO_4^{2-}, \quad E^o(SO_4^{-\cdot}/SO_4^{2-}) = 3.4$ V vs. SHE

Hydrogen peroxide and peroxodiphosphate ions can also be used in analogous manner in generation of strongly oxidising radicals (reactions 8c-8d).

(8c) $e_{aq}^- + P_2O_8^{4-} \rightarrow PO_4^{\cdot 2-} + PO_4^{3-}, \quad E^o(PO_4^{\cdot 2-}/PO_4^{3-}) = 2.5$ V vs. SHE

(8d) $e_{aq}^- + H_2O_2 \rightarrow OH\cdot + OH^-, \quad E^o(\cdot OH/OH^-) = 2.7$ V vs. SHE

Analogously, LL process can be initiated by dissolving solid that exclusively produces oxidising species in its dissolution reaction. In some cases, the solvent itself can act as a reductant or oxidant (reactions 9a-9c). A reducing species can be also generated from solutes such as, formiate ion or oxalate ion or from tertiary, secondary or primary amines which produce strongly reducing species at appropriate pH range upon one-electron oxidation.

(9) UV- irradiated $K_2S_2O_8 \xrightarrow{H_2O} 2\ SO_4^{-\cdot} + 2\ K^+$

(9a) $Tb(III) + SO_4^{-\cdot} \rightarrow Tb(IV) + SO_4^{2-}$

(9b) $Tb(IV) + water \rightarrow Tb(III)^*$

(9c) $Tb(III)^* \rightarrow Tb(III) + h\nu$

Some label substances, such as derivatives of isoluminol or luminol, can be excited by producing strongly oxidising species, such as sulfate, phosphate, hydroxyl, or halide containing radicals in the vicinity of labels so that the simultaneous presence of a strong reductant is unnecessary (10a-10b).

(10a) $Luminophore + n\ Ox^{\cdot} \rightarrow intermediates + n\ Ox^-$

(10b) $intermediates \rightarrow products + h\nu$

In the aforementioned equations, $Ox^{\cdot}$ is one of the said strongly oxidising one-electron oxidants and luminophore is, e.g., a derivative of luminol or isoluminol. For example, in strongly oxidising conditions, luminol produces several intermediates and finally 3-aminopthalate in its excited state which is the emitter of the system.

Strongly reducing metals can be used to produce extrinsic LL. This can be carried out, e.g. by exposing a fresh metal surface in contact with an aqueous solution by dissolving the surface oxide film of the metal with acid or base, after which the metal surface or low-valent short-lived metal, such as Mg(I), Al(I) and Al(II), can act as reducing mediators at distances longer than is possible by reactions induced by electron or hole tunnelling from the metal/solution interface. Normally, the reductants must produce secondary oxidising species from coreactants such peroxodisulfate, peroxodiphosphate or hydrogen peroxide according to analogous reaction to reactions (8b)-(8d) where hydrated electron can be replaced by atomic hydrogen, reducing metal, or low-valent metal ion.

The energy released in the above-mentioned dissolution process is transformed into light by redox reactions (or energy transfer) of label and the primary dissolution products. The analytical method itself is based on the measurement of the amount of label molecules. The dependence between label molecules and the analyte can be accomplished by known methods, such as, methods based on bioaffinity (see e.g. The Immunoassay Handbook, Edited by David Wild, Stockholm Press Ltd., New York, 1994, pp 1-618). The label compound can be a luminescent metal chelate or an organic molecule or a polymer particle containing luminophores, which is bound to bioactive molecule by a linking molecule. According to the invention, when the dissolution is standardised, the same amount of label molecules generates the same amount of photons.

The excitation is usually done in the luminometer so that a disposable test tube contains constant amount LL-producing solid material, and the label material released from a solid support, or a suspension of latex particles, when these are used as a solid support, is injected on the salt while the light is being measured. The paramagnetic latex beads are especially advantageous, because all washings can be done very simply and fast. There are also other alternatives. For example, peroxonitrite formed from potassium nitrate under UV-irradiation is very stable in basic solutions (0.1 M NaOH). Therefore, this dissolution product can be treated in the same manner as any other liquid reagent of chemiluminescence and the process of chemiluminescence can be triggered by addition

of acid. The LL-producing material can also be dried as a film inside the reaction well before irradiation, then LL-producing thin films can be prepared, or LL-producing powder can be sampled into the liquid flow by revolver type device.

The lyoluminescence can be measured depending on the required sensitivity range either by using a low-cost semiconductor detector or in the case of an analyte requiring extreme sensitivity, a photomultiplier tube. In case no electricity is available, the instrument can be equipped with battery, accumulator, or the instrument involves a sensitive photographic film. Because differences in the developing processes can occur, a standard light source can be needed as a reference, and this reference light source can be, for example, a tritium lamp. It is composed of tritium containing glass capsule, where the inner side is covered by luminescent layer, which is excited by tritium and gives constant light. The glass capsule prevents the β-radiation entering outside of the capsule, therefore, this standard light source is also safe and therefore it is in use, e.g., as a component of the night viewfinder in hand arms. The intensity of the light coming from the tritium lamp to the film can be standardised to a required level by optical filters and/or by adjusting the distance. When photographic film is used as a detector, the exact result can be read from the film by using a special densitometer, but in the case that the sensitivity of a Polaroid film is sufficient in on-off tests, the result of the analysis can be read immediately without the help of a photographic laboratory by comparing the colour intensity of the sample spot with the colour intensity of two others colour spots produced by standard light sources giving upper and lower limits for the assay. The highest sensitivity is achieved by using X-ray film or by other sensitive films (e.g. Kodak 3200 ASA), but in these cases, it is necessary to develop the film in a photographic laboratory. An advantage is, however, that a fresh sample can be used and only the film has to sent to be developed. The result in field analysis is still reliable, although the result is not immediately available after the analysis. Especially biological samples are not stable and this creates variation and errors in results. The photographic film can be replaced also by battery-operated CCD or a digital camera. Then the results can be obtained very fast by a computer equipped with appropriate software.

According to this invention, suitable compounds for labelling are, e.g., the derivatives of the following organic luminophores: 9-fluorenylmethylcloroformate (emission 309 nm), luminol (emission 420 nm), fluorescein (emission 516 nm), salicylate derivatives (emission in the rage of 400-450 nm), derivatives of aminonaphthalene sulphonic acid (emission in the range of 400-500 nm) and coumarines (emission in the range of 450-550 nm), derivatives of aromatic lanthanide (III) chelates, such as certain derivatives of terbium (III) complexes with the following ligands: N^1-(4-aminobenzyl)diethylenetriamine-N^1,N^2,N^3,N^3-tetra-acetate (abbr. Tb(III)-**1**), 4-(phenylethyl)(1-hydroxybenze-ne)-2,6-diyl)bismethylenenitrilo)tetrakis(acetate) (Tb(III)-**2**); 4-benzoyl(1-hydroxybenzene)-2,6-diyl)-bis(methylenenitrilo)tetrakis(acetate) (Tb(III)-**3**), N^2-(4-aminobezyl)diethylenetriamine-(N^1,N^1,N^3,N^3,-tetra-acetate (Tb(III)-**4**); 4-methyl(1-hydroxybenzene)-2,6-diyl(bis(methylenenitrilo)tetrakis(acetate) (Tb(III)-**5**) (the strongest emission line at 545 nm in the cases of all Tb(III) chelates), derivatives of certain transition metal chelates such as ruthenium(II) and osmium(II)-trisbipyridyl and trispyratsyl complexes (emission in the range of 550-650 nm).

Some chemical reactions involved in the LL mechanism are described in the explanation part of the present invention, but the method according to the invention can also be based on other chemical processes related to the dissolution of solid material, by which the label is excited. The structure of the label can also be varied so, that the labels can emit light from the range of UV-light to the range of infrared light and the instrument can also vary accordingly. The LL-excitation itself can be produced in practice by many ways depending the instrument set-up in use.

Summary of the invention

A method, wherein the excitation of label molecules is achieved by a dissolution of a solid material in a solvent followed by exploitation of the resulting luminescence for chemical and biochemical analyses. In the said process intermediates responsible for the excitation of labels are either dissolution-released and/or produced from the solid or solvent during dissolution, or produced from coreactants added in the solvent. Either one or several

different label compounds can be excited to excited state(s) which is(are) deexcited by emission of ultraviolet, visible or infrared light providing the basis for reproducible analytical applications in bioaffinity assays such as in immunoassays and DNA-probing assays.

Brief description of the drawings

Figure 1. Diagram of a lyoluminometer. (1) Test tube or cell, which contains solid material able to produce lyoluminescence. (2) Pipe through which the solution or suspension of label is injected to the cell of lyoluminometer. (3) Optical filter, the choice of which is dependent on the emission spectrum of the label.

Figure 2. Diagram of a lyoluminometer without any electrical components. (1) Light-tight box. (2) Light-sensitive film. (3) Test tube or cell, which contains solid material able to produce lyoluminescence. (4) Tube through which the solution or latex bead suspension is injected to the cell of lyoluminometer. (5) Tritium lamp 1. (6) Tritium lamp 2. (7) Adjusting screw for the distance of tritium lamp 1. (8) Adjusting screw for the distance of tritium lamp 2. (9) Neutral density filter 1. (10) Neutral density filter 2.

Figure 3. Detection of N-(6-aminobutyl)-N-ethylisoluminol (ABEI) by different methods of lyoluminescence. (a) Additively coloured KCl, pH 11.5, $3x10^{-4}$ M $K_2S_2O_8$ (open circle, dashed line). (b) Electrolytically coloured KCl, pH 11.5, $3x10^{-4}$ M $K_2S_2O_8$(open square, dashed line). (c) UV-irradiated $K_2S_2O_8$, pH 12 (circle). (d) UV-irradiated $K_4P_2O_8$, (diamond), (e) UV-irradiated KNO_3, pH 11 (square), (f) Aluminium, pH 12, 0.03 M $K_4P_2O_8$.

Figure 4. Lyoluminometric immunoassay of $ß_2$-microglobulin using latex beads when the detection of the label (AHEI) is based on LL produced by UV-radiated $K_2S_2O_8$, $K_4P_2O_8$, or KNO_3.

Figure 5. Lyoluminometric immunoassay of $ß_2$-microglobulin on the inner surface of aluminium cup (a), on the surface of aluminium particles (b), or on latex beads (c) wherein the detection of the label (AHEI) is based on the LL of aluminium in alkaline conditions.

Figure 6. Lyoluminometric immunoassay of $ß_2$-microglobulin on the inner surface of aluminium cup when the detection is based on the LL produced by electrolytically alkalised aluminium electrode. The label is an isothiocyanate derivative of Tb(III) chelate.

Figure 7. Lyoluminometric immunoassay of human thyrotropin (TSH) on latex beads. The detection was based on extrinsic LL produced by magnesium in acidic conditions (a), by aluminium in alkaline conditions (b), or by additively coloured KCl (c). The label was a derivative of $Ru(bpy)_3^{2+}$-chelate.

Figure 8. Lyoluminometric immunoassay of phospholipase A_2 on latex beads when the detection is based on LL produced by additively coloured KCl and the label is an isothiocyanate derivative of Tb(III) chelate.

Figure 9. Lyoluminometric immunoassay of phospholipase A_2 on latex beads. The detection was based on extrinsic LL produced by additively coloured KCl while the label was AHEI.

Figure 10. Lyoluminometric immunoassay of phospholipase A_2 on latex beads. The detection was based on extrinsic LL produced by aluminium in the presence of $P_2O_8^{4-}$ -ions. The label was alkaline phosphatase with phosphate esther of 5-fluorosalicylic acid as substrate. (a) The product of the reaction was detected directly. (b) The product of the substrate was allowed to form ternary complex with Tb(III)-EDTA before detection.

Figure 11. Lyoluminometric immunoassay of $ß_2$-microglobulin on microtitration plates. After immunochemical reaction Tb(III)-ion was detached from the antibody and the

released Tb(III)-ion was measured as a new complex. The detection was based on extrinsic LL produced by UV-irradiated $K_2S_2O_8$ or additively coloured KCl while the label was an isothiocyanate derivative of a Tb(III) chelate.

Figure 12. Standard curve of a lyoluminometric immunoassay of $ß_2$-microglobulin. Liposomes containing luminophores were used as the label while the detection is based on LL produced by electrolytically coloured KCl in the presence of $S_2O_8^{2-}$ ions.

Figure 13. Standard curve of a lyoluminometric immunoassay of $ß_2$-microglobulin. The label was a luminophore, which can be released with UV-light while the detection was based on an extrinsic LL produced by additively coloured KCl.

Figure 14. Lyoluminometric immunoassay of $ß_2$-microglobulin. The assay was based on the release of label from solid support before producing LL by UV-irradiated potassium peroxodisulfate.

Figure 15. Detection of Philadelphia chromosome lyoluminometrically by DNA-hybridisation method. The label was an isothiocyanate derivative of Tb(III) chelate.

Example 1. *Preparation of energetic salts and calibration curves of N-(6-aminobutyl)-N-ethylisoluminol generated by different LL-methods.*

Additive coloration of potassium chloride. Microcrystal KCl (500 mg, Merck, Suprapur) was coloured additively at the pressure of 15 torr of potassium vapour at the temperature of 630° C for 12 h.

Electrolytic coloration of potassium chloride. KCl (Merck, Suprapur) was dried first by heating at 500° C in vacuum and then one crystal was grown by the method of Czochralsky in argon atmosphere. A piece of crystal (12x12x20 mm) was coloured electrolytically by the matrix cathode method at 700° C and current 1 mA.

UV-irradiation of potassium peroxodisulfate, potassium peroxodiphosphate and potassium nitrate. Each salt was spread by turn on the surface of glass as a thin layer (thickness of one crystal) and the radiated by a UV-lamp of Philips TUV/15W, G15T8 at the distance of 6 cm for 4 h. Salt was then collected in a test tube and homogenized by shaking.

Convective injection. It is possible to perform with a normal laboratory precision pipette reproducible injections by choosing correctly the diameter of the injection tubing and the geometry of the outlet end of the tubing and the shape of the LL-cell, so that the convection in the liquid in the cell is the same from one injection to another. Preferably, the injection is performed by a pipette with battery-operated step motor, which has adjustable speed of injection.

Calibration curves of ABEI. (a) Additively coloured KCl (15.0 mg) was weighed into a disposable test tube (1.2 mL) and then ABEI solution (1 mL) was injected into the tube in 3 mM NaOH solution containing $3x10^{-4}$ mol/L potassium peroxodisulfate (open circle, dashed line). (b) Electrolytically coloured KCl (15 mg), ground into fine powder in agate mortar after coloration, was weighed into a disposable test tube (1.2 mL) and 1 mL of ABEI solution in 3 mM NaOH solution containing $3x10^{-4}$ mol/L potassium peroxodisulfate (open square, dashed line). (c) UV-irradiated $K_2S_2O_8$ (10.0 mg) was weighed into a disposable test tube (1.2 mL) and then ABEI solution (1 mL) was injected into the tube in 0.01 M NaOH solution (circle). (d) UV-irradiated $K_4P_2O_8$ (10.0 mg) was weighed into a disposable test tube (1.2 mL) and then ABEI solution (1.00 mL) was injected into the tube in 0.01 M NaOH solution (diamond). (e) UV-irradiated KNO_3 (10.0 mg) was weighed into a disposable test tube (1.2 mL) and then ABEI solution (1.00 mL) was injected into the tube in 1 mM NaOH solution (square). (f) Luminol solution (1.0 mL) in 0.01 M NaOH solution containing 0.03 mol/L $K_4P_2O_8$ was injected into an aluminium cup. All measurements were done with a lyoluminometer based on photon counting (S. Kulmala and K. Haapakka, Acta Chimica Acta, 249, 1994, 13.), where the structure and geometry of the injection tube and thus the hydrodynamics was

redesigned. The 546-nm interference filter was replaced by 450-nm interference filter. The measured calibration curves are shown in Figure 3.

Example 2. *Lyoluminometric determination of β_2-microglobulin using N-(6-aminohexyl)-N-ethyl-isoluminol as label. LL was produced with UV-saturated potassium peroxodisulfate.*

Coating latex particles with antibody. The stock suspension of latex particles (Sigma, LB-8) was diluted to 1:100 in TSA buffer (0.05mol/L TRIS-HCl, pH 7.75, 0.9% NaCl, 0.05% NaN_3). To 100 microlitres of this dilution was added 100 µL of solution which contained 6 mg/mL mouse anti-β_2-microglobulin antibody (clone 6G12, Labmaster Ltd., Turku) in TSA buffer and the mixed solution was incubated overnight. The particles were separated from supernatant by centrifugation and washed with wash solution (0.01 M TRIS-HCl, pH 7.75, 0.9% NaCl, and 0.02% Tween 20). After this, the latex particles were saturated overnight in a TSA buffer containing 0.1% bovine serum albumin (BSA).

Preparing of labelled antibody. The other mouse anti-β_2-microglobulin antibody (clone 1F10, Labmaster Ltd., Turku, Finland) was labelled with N-(6-aminohexyl)-N-ethyl-isoluminol (AHEI) according to Schroeder et al. (*Methods in Enzymology*, Vol 57, M. DeLuca (edit), Academic Press, N.Y., 1978).

β_2-microglobulin standard. Standards (0.4, 1.6, 4.0, 8.0, and 16 mg/L) were prepared from β_2-microglobulin purified from human ascites fluid (75.5 mg/mL, Labmaster Ltd., Turku, Finland) into the TSA buffer. TSA buffer was supplemented by 7.5-% BSA.

Immunochemical detection. Twenty µL of suspension (diluted 1:20) of coated latex particles (about 180 million latex particles) were added to centrifugal tubes (volume 1.5 mL; saturated with BSA) made of polypropylene. Standards were diluted 1:50 with the assay buffer (0.05 mol/L TRIS-HCl, pH 7.75, containing 0.9% NaCl, 0.05% NaN_3, 0.5% BSA and 0.01% Tween 20) and were added onto the bottom of the cuvette (40 µL). Then 120 µL of labelled antibody was added and the solution was incubated for 1 hour at room

temperature with shaking. After that the particles were separated with a centrifuge they were washed three times with the wash solution and once with distilled water containing 0.02% Tween 40 (p/p). Finally the particles were suspended into the LL measurement solution (0.01 M NaOH) and injected to the measurement cuvette containing 10.0 mg of UV-irradiated $K_2S_2O_8$. LL was measured as in Example 1e. The standard curve is shown in Fig. 4.

Example 3. *Lyoluminometric determination of β_2-microglobulin using AHEI as label. The LL was produced by dissolving aluminium with NaOH in the presence of $P_2O_8^{4-}$ ions.*

Coating aluminium cup with antibody. Aluminium cups (capacity 500 µL) were pressed from aluminium plate (thickness 0.3 mm). Cups were coated with mouse anti-β_2-microglobulin antibody (clone 6G12, Labmaster Ltd., Turku) by incubating 500 µL antibody (10 mg/mL) in 0.2 M NaH_2PO_4 solution in the cup over night. The next day the electrode was washed six times with the wash solution and equilibrated overnight in the saturation solution (0.05 M TRIS-HCl, pH 7.75 containing 0.1 % BSA, 6 % of sorbitol and 1 mmol/L $CaCl_2$). Aluminium cup was then aspirated empty and stored dry.

Coating aluminium powder with antibody. Aluminium powder was coated with mouse anti-β_2-microglobulin antibody (clone 6G12, Labmaster Ltd., Turku, Finland) in the same way as latex particles in Example 2.

Preparing of labelled antibody. The other mouse anti-β_2-microglobulin antibody (clone 1F10, Labmaster Ltd., Turku, Finland) was labelled with AHEI as in Example 2.

β_2-microglobulin standard. Standards were prepared as in Example 2.

Immunochemical determination with aluminium cups. Standards were diluted 1:50 with the assay buffer (0.05 mol/L TRIS-HCl, pH 7.75, containing 0.9% NaCl, 0.05% NaN_3, 0.5% BSA and 0.01% Tween 20) and were added onto the bottom of the aluminium cup

(40 µL). Then 120 µL of labelled antibody was added and the solution was incubated for an hour at room temperature with shaking. After this, the cups were washed four times with the wash solution and once with distilled water. Finally the cups were transferred one by one into the lyoluminometer. To each of the aluminium cups 400 µL 0.01 M NaOH solution containing 0.03 mol/L $K_4P_2O_8$ was injected. The light was integrated during 300 seconds. The measured calibration curve is shown in Fig. 5, curve (a).

Immunochemical determination using aluminium powder. Thirty µL of suspension (diluted 1:20) of coated aluminium powder were added to the centrifuge tubes (volume 1.5 mL) made of polypropylene. The tubes were presaturated with BSA. Standards were diluted 1:50 with the assay buffer (0.05 mol/L TRIS-HCl, pH 7.75, containing 0.9% NaCl, 0.05% NaN_3, 0.5% BSA and 0.01% Tween 20) and were added onto the bottom of the cuvette (40 µL). Then 120 µL of labelled antibody in the assay buffer were added and the solution was incubated for an hour at room temperature with shaking. After this, the aluminium powder was separated by centrifugation and washed three times with the wash solution and once with distilled water containing 0.02% Tween 40 (p/p). The test tube was sucked dry and transferred into the LL compartment. Finally 400 µL 0.01 M NaOH solution containing 0.03 mol/L $K_4P_2O_8$ was injected to the test tube. The light was integrated during 300 seconds. The measured calibration curve is shown in Fig. 5, curve (b).

Immunoassay with latex particles. The assay was performed as in Example 3, except that the particles were finally washed with distilled water containing 0.02 % Tween 40 (p/p). Afterwards, the particles were suspended into 400 µL of 0.01 M NaOH solution containing 0.03 mol/L of $K_4P_2O_8$. The light was integrated during 300 seconds. The measured calibration curve is shown in Fig. 5, curve (c).

Example 4. *Lyoluminometric immunoassay of β_2-microglobulin on the inner surface of Aluminium cups using an isotiocyanate derivative of a Tb(III) chelate as label. LL was produced by electrolytic cathodisation of aluminium in the presence of $S_2O_8^{2-}$ ions.*

Aluminium cups were coated with antibody as in Example 3.

Preparing labelled antibody. A mouse anti-β_2-microglobulin antibody (clone 1F10, Labmaster Ltd., Turku, Finland) was labelled with the isothiocyanate derivative of Tb(III)-**4** chelate [Tb^{3+} -N^2-(4-isothiocyanatobetzyl)-diethylenetriamine-N^1,N^1,N^3,N^3-tetra acetate] (Wallac Ltd., Turku). The antibody was allowed to react with chelate in molar ratio of 1:60 at pH 9.5. The pH was adjusted with 1 M Na_2CO_3. The labelled antibody was purified from unreacted chelate by gel filtration (Sepharose 6B 1 x 50 cm, Sephadex G-50 1 x 5 cm) using TSA buffer (0.05 mol/L TRIS-HCl, pH 7.75, 0.9% NaCl, 0.05% NaN_3) as the mobile phase. Typically, 5-10 chelate molecules can be bound to one antibody molecule with this procedure. To improve the stability, 0.1% of BSA was added into the solution of labelled antibody.

Immunoassay with aluminium cups was done as in Example 3. After this, 0.5 M Na_2SO_4 containing 0.01mol/L $K_2S_2O_8$ was added to the aluminium cups. The platinum wire was placed as counter electrode into the cell and electrolysis was started with 10 V DC voltage so that the aluminium electrode served as the cathode. The light was measured during 300 seconds and the calibration curve is shown in Fig. 6.

Example 5. *Lyoluminometric immunoassay of TSH. LL was produced by dissolving magnesium in HCl, aluminium in NaOH, or by additively coloured KCl in an aqueous solution containing $S_2O_8^{2-}$ ions.*

Paramagnetic latex particles coated with streptavidin, monoclonal anti-TSH antibody labelled with $Ru(bpy)_3^{2+}$ and biotinylated monoclonal anti-TSH antibody were products of Boehringer Mannheim (Elecsys TSH Immunoassay Kit, Germany).

Preparing standards. Standards were diluted from the stock solution of TSH (52300 μU/mL, Wallac Ltd., Turku, Finland) to the dilution buffer (Labmaster Ltd., Turku, Finland). The concentrations of standards were 9.0, 54.0 and 324 μU/mL.

Lyoluminometric immunoassay. Immunochemical determination was performed according to the instructions by Boehringer Mannheim, except that 50 µL of standards were added to the test tubes and the volume of solution of anti-TSH antibody labelled with $Ru(bpy)_3^{2+}$ was 100 µL and the volume of biotinylated monoclonal anti-TSH antibody was also 100 µL. During the first 25 minutes the incubation was done with shaking. After this, incubation 200 µL of newly-shaked suspension of streptavidin-coated magnetic latex particles were added and the mixture was shaked for 10 minutes. After the washing procedures done as recommended by the manufacturer the particles were additionally washed once with distilled water containing 0.02% of Tween 40 (p/p). Finally particles were suspended in the measurement solution of each experiment.

With the LL system produced by magnesium, the particles were suspended into a 1.00 mL of 0.1 M HCl containing 0.005% Tween 40. The suspension was injected to the test tube where a 1.0-cm piece of Mg band (Merck Art. No. 5812) was pressed on the bottom. The LL was integrated during 400 seconds through a 620-nm interference filter. Standard curve is shown in Fig. 7, curve (a). With the LL system produced by aluminium, the particles were suspended into a 1.00 mL aliquot of 0.1 M NaOH containing 0.005% Tween 40 and 0.01 mol/L $K_2S_2O_8$. The suspension was injected into the aluminium cup inside the lyoluminometer and the LL was integrated during 600 seconds. Standard curve is shown in Fig. 7, curve (b).

With the LL system produced by additively colored KCl, the particles were suspended into a 1.00 mL of 0.2 M borate buffer containing 0.005% Tween 20 and $3x10^{-4}$ mol/L $K_2S_2O_8$. The suspension was injected into a test tube containing 25.0 mg of additively coloured KCl powder. The LL was integrated 10.0 s starting at the injection point. Standard curve is shown in Fig. 7, curve (c).

Example 6. *Lyoluminometric assay of phospholipase A_2 (PLA_2) with latex particles. LL was produced by additively coloured KCl with an isothiocyanate derivative of Tb(III)-chelate as the label.*

Coating of latex particles with antibodies. Stock suspension of latex particles (Sigma, LB-8) was diluted to 1:100 into a TSA-buffer (0.05 mol/L Tris-HCl, pH 7.75, 0.9% NaCl, and 0.05% NaN_3). To this dilution (100 µL), 100 µL of a solution containing 5.7 mg/mL of anti-PLA_2 antibody (clone 2E1, Labmaster, Turku, Finland) in TSA buffer, were added and then incubated overnight. Particles were separated by centrifugation and washed with the wash solution. Then latex particles were saturated overnight in a TSA buffer containing 0.1 % of BSA.

Preparation of labelled antibody. Sheep polyclonal antibody against human pancreatic phospholipase A_2 (affinity purified; Labmaster Oy, Turku, Finland) was labelled with an isothiocyanate derivative of Tb(III)-**1** chelate [Tb^{3+}- N-(*p*-isothiocyanatobentsyl)-diethylentriamine-N^1, N^2, N^3,-tetra-acetate; Wallac Oy, Turku, Finland], allowing the antibody to react with the chelate in molar ratio of 1:60 at pH 9.5. The pH was adjusted with 1 M Na_2CO_3-solution. The labelled antibody was purified from unreacted chelate with gel filtration (Sepharose 6B 1 x 50 cm and Sephadex G-50 1 x 5 cm on the top of the column) using TSA-buffer as the mobile phase. Typically, 5 -10 chelate molecules were bound to one antibody. BSA (0.1%) was added to the product to improve the stability of the labelled antibody.

Preparation of the standards. Human pancreatic PLA_2 (Labmaster Oy, Turku, Finland) was dissolved in TSA-buffer, containing 7 % (w/v) BSA, to obtain concentrations of 0, 1.5, 9, 54, and 324 ng/mL.

Immunoassay. A dilution (1:20, about 180 million particles) of antibody-coated latex particle suspension (20 µl) was taken into 1.5-mL polypropen centrifugal tubes saturated with a BSA solution. To this suspension, 20 µL of standard and 20 µL of Tb(III)-**1**-labelled antibody (500 ng) were added and the mixture was incubated for 20 min at room

temperature. Then the particles were separated with centrifugation and washed twice with the wash solution and once with the LL-measuring buffer (0.2 M borate buffer, pH 7.3, including 0.02 w/w-% of TWEEN 40). The particles were suspended in the LL-measuring buffer and LL was measured as in Example 1 (a), except that án interference filter of 546 nm was employed. In this case LL-measuring buffer contained 0.2 M borate, pH 7.3, with $3x10^{-4}$ M $K_2S_2O_8$ and 0.02 w/w-% TWEEN 40. The suspension was injected inside a lyoluminometer into a test tube containing 20.0 mg of additively coloured KCl. Standard curve is shown in Fig. 8.

Example 7. *Lyoluminometric immunoassay of phospholipase A_2 using latex particles. LL was produced with electrolytically coloured KCl.*

The same antibody-coated latex particles and the same standards as in Example 6 were applied in the experiment.

Preparation of labelled antibody. Sheep polyclonal antibody against human pancreatic PLA_2 (affinity purified; Labmaster Oy, Turku, Finland) was labelled with AHEI and was purified as in Example 2.

The immunoassay was carried out as in Example 6 except that the latex particles were suspended finally in an 0.01-M NaOH solution, containing 1 mmol/L $K_4P_2O_8$. The suspension was injected immediately into a test tube placed inside a lyoluminometer (450 nm interference filter). The test tube contained 20.0 mg of powder of electrolytically coloured KCl. Standard curve is shown in Fig. 9.

Example 8. *Lyoluminometric assay of PLA_2 using latex particles as solid carriers in a method exploiting enzymatic signal amplification. LL was produced with additively coloured KCl or with UV-irradiated $K_2S_2O_8$.*

Latex particles were coated as in Example 6.

Labelling of antibodies with alkaline phosphatase. Sheep polyclonal anti-PLA_2 antibody was labelled with alkaline phosphatase (ALF) with the maleimide method (E. Ishikawa, M. Imagawa, S. Hashida, S. Yoshitake, Y. Hamuguchi and T. Ueno, *J. Immunoassay,* 1983, **4**, 209).

Immunoassay. A dilution (1:20; about 180 million particles) of antibody-coated latex particle suspension (20 µl) was taken into 1.5-mL polypropen centrifugal tubes saturated with a BSA solution. To this suspension, 20 µL of standard and 20 µl of ALF-labelled antibody (600 ng) were added and the mixture was incubated for 15 min at room temperature. The particles were separated by centrifugation and washed twice with the wash solution. The particles were suspended in 200 µL of a solution containing 1 mmol/L of substrate (phosphate ester of 5-fluorosalicylic acid, FSAF; Kronem Systems Inc. Mississauga, Ontario, Canada). Supernatant was separated after a 15-min incubation and a 100-µL portion of supernatant was transferred into a test tube containing 0.900 mL of 0.01 M $K_2S_2O_{82}$ in 0.5 M NaOH. The solution was injected into an aluminium cup and LL was measured through a 420-nm interference filter. A standard curve (a) of Fig. 10 was obtained. Alternatively, the detection was carried out as follows: 150 µL of the supernatant was added to 850 µL of 0.5 mM Tb(III)-EDTA solution in 0.2 M NaOH. The solution was stirred with a pipette and incubated for 15 min. Into this solution, 25 µL of 0.01 M $K_2S_2O_8$ were added and the stirred solution was injected into an aluminium cup. LL-signal was measured during 300 s at 545 nm (Fig. 10 b).

Example 9. *Lyoluminometric immunoassay of β_2-microglobulin on microtiter plates with detaching Tb-ion after the immunoreaction from antibody and measuring the released Tb ions with a new complex excited by UV-irradiated $K_2S_2O_8$ or additively coloured KCl.*

Coating of microtiter plates with antibody. Mouse anti-β_2-microglobulin antibody (200 µL, 10 mg/mL; clone 6G12, Labmaster Oy, Turku, Finland) was pipetted into wells of microtiter plates. Next day the wells were washed six times with a wash solution (0.01 M Tris-HCl, pH 7.75, 0.9 % NaCl and 0.02 w/w-% Tween 20) and equilibrated with a

solution (200 mL/well) of 0.05 M Tris-HCl, pH 7.75, 1 g of BSA, 60 g sorbitol and 1 mmol $CaCl_2$ in litre of solution. Then the wells were aspirated empty and were stored as wet.

The antibody was labelled with an isothiocyanate derivative of Tb(III)-**1** chelate as described in Example 4.

Immunoassay. Standards were diluted into the assay buffer to 1:50 (buffer was 0.05 M Tris-HCl, pH 7.75, with 0.9 % of NaCl, 0.05 % NaN_3, 0.5 % BSA and 0.01 w/w-% Tween 20) and the standards (40 µL) were pipetted into the wells. Then 160 µL of labelled antibody (500 ng, clone 1F10, Labmaster Oy, Turku, Finland) in assay buffer were added. The immunoreaction was allowed to take place and after one hour the wells were washed six times with the wash solution followed by addition of 200 µL of 0.1 M glycine-H_2SO_4-buffer, pH 2.5, and then incubated for 15 min. A portion of this solution (150 µL) was taken into the measuring cuvette and 50 µL of 0.5 M Na_2SO_4 containing $5x10^{-4}$ M ligand **5** were added. The solution was stirred and 800 µL of 0.2 M $Na_2B_4O_7$ -buffer were added. The Tb(III)-**5** chelate was quantified by its injection into a test tube (inside a lyoluminometer; 546-nm interference filter) containing 10.0 mg of UV-irradiated $K_2S_2O_8$. Standard curve is shown in Fig. 11.

Example 10. *Immunoassay of β_2-microglobulin exploiting liposomes and electrolytically coloured KCl and $S_2O_8^{2-}$ ions.*

Liposomes containing Tb-**5**-complex were prepared first. They were bound to an antibody (anti-β_2-microglobulin, clone 6G12, Labmaster Oy, Turku, Finland) as described by GP. Vonk, B. Wagner, Clinical Chemistry, 1991, 37, 1519. The immunoreaction was carried out as in Example 9, except that no surfactant was used. After washings, 230 µL of 0.1 w/w-% Triton X-100 solution in 0.1 M glycine buffer (pH 3.2) were added and incubated for 10 min. A portion of the solution (200 µL) was taken into a test tube and 60 µL of 0.5 M Na_2CO_3 containing $1x10^{-3}$ M of ligand **5**. Finally, 0.800 mL of 0.05 M of $Na_2B_4O_7$

buffer were added, stirred and injected into a test tube (inside a lyoluminometer; 545- nm interference filter) containing 20.0 mg of finely ground electrolytically coloured KCl. Standard curve is shown in Fig. 12.

Example 11. *Immunoassay of β_2-microglobulin with using a label detached by UV-irradiation and additively coloured KCl in the presence of $S_2O_8^{2-}$ ions.*

The wells of microtiter plates were coated as in Example 9.

Labelling of antibody. An antibody (clone 1F10, Labmaster Oy, Turku, Finland) was labelled with a UV-detachable label (Rhodamine Green sulfosuccinimidyl ester; Molecular Probes, R-7091, Eugene, USA) according to the manufacturer's instruction.

The immunoassay was performed on microtiter plates as in Example 9, except that after the washing, 200 µL of 0.05 M $Na_2B_4O_7$ were pipetted into the wells.

Detachment of label with UV light and LL measurement. Microtiter plate was exposed to UV radiation (Philips HPLR UV-source) for 4.0 min from the top. From each well, 180 µL of solution was taken into a small test tube. Into it, 820 µL of 0.05 M $Na_2B_4O_7$ containing $5x10^{-4}$ M $K_2S_2O_8$ was pipetted and the stirred mixture was injected into a test tube (in a lyoluminometer) containing 30.0 mg additively coloured KCl. Lyoluminometer involved a 500-nm bandpass filter. Standard curve is shown in Fig. 14.

Example 12. *Immunoassay of β_2-microglobulin exploiting AHEI as the label after its detaching from solid support with chaotropic agents. The LL was produced by UV-irradiated peroxodisulfate.*

Coating of microtiter plates with antibodies. Mouse anti-β_2-microglobulin antibody (200 µL; 10 mg/mL, clone 6G12, Labmaster Oy, Turku, Finland) in 0.2 M NaH_2PO_4 was pipetted into the wells of a microtiter plate. Next day the wells were washed six times with

a wash solution (0.01 M Tris-HCl, pH 7.75, 0.9 % NaCl and 0,02 w/w-% Tween 20) and were equilibrated each with 200 µl of solution containing 0.05 M Tris-HCl, pH 7.75, 0.1 % BSA, 6 % sorbitol and 1 mM $CaCl_2$. The wells were then aspirated empty and stored as wet.

Preparation of labelled antibody. Mouse anti-$ß_2$-microglobulin antibody (clone 1F10, Labmaster Oy, Turku, Finland) was labelled with N-(6-aminohexyl)-N-ethyl-isoluminol (AHEI) by the method of Schroeder et al. (*Methods in Enzymology*, Vol 57, M. DeLuca (Ed.), Academic Press, N.Y., 1978).

$ß_2$-microglobulin standards. Standards were made of human $ß_2$-microglobulin (75.5 mg/mL, Labmaster Oy, Turku, Finland) purified from human ascites, fluid into a TSA-buffer with 7.5 % BSA, in concentrations of 0.4, 1.6, 4.0, 8.0 and 16 mg/L.

Immunoassay. Standards were diluted in assay buffer (0.05 mol/L Tris-HCl, pH 7.75, including 0.9 % NaCl, 0.05% NaN_3, 0.5 % BSA, and 0.01 % Tween 20) to 1:50 and standards (40 µL) were pipetted into wells. Then, 160 µL of labelled antibody (500 ng; clone 1F10, Labmaster Oy, Turku, Finland) in assay buffer were added. Immunoreaction was allowed to occur for one hour and the wells were washed six times with the wash solution. The labelled antibody bound to the surface of the wells was detached chaotropically with 0.2 M sodium borate, pH 9.2, containing 1.75 mol/L NaSCN (250 µL). After incubation, 200 µL of the solution was transferred into a test tube containing 800 µL 0.2 M sodium borate, pH 9.2, and stirred. The mixture was injected into a test tube (in lyoluminometer; 450-nm interference filter) containing 10.0 mg of UV-irradiated $K_2S_2O_8$ and then LL was determined as in Example 2. Standard curve is shown in Fig. 14. Alternatively, labelled antibody or antigen can be detached from solid support as in the Finnish patent number 88545.

Example 13. *Detection of Philadelphia chromosome with DNA-hybridisation method exploiting latex particles and UV-irradiated $K_2S_2O_8$.*

Labelling of DNA-probe with Tb-chelate. An oligonucleotide containing amino groups (TTCGGGAAGTCGCCGGTCATCGTAGA-(C-NH_2)$_{25}$-5'; Wallac Oy, Turku, Finland) were labelled with a Tb-chelate as in Example 4, except that the probe was purified by gel filtration on NAP-5 and NAP-10 columns (Pharmacia, Uppsala, Sweden).

Labelling of oligonucleotide with biotin. Another biotin-labelled probe of aminated oligonucleotide (C-(NH_2-C)-GTCGTAAGGCGACTGGTAGTTATTCCTT-5'; Wallac, Turku, Finland), was also prepared for DNA hybridisation assay. An N-hydroxysuccinimide-ester-derivative of biotin in 3.7 µL of N,N-dimethylformamide was allowed to react overnight with the oligonucleotide (5 nmol in 50 µL, with molar ratio of 50:1) at pH 9.5 at +4°C. The pH was adjusted by adding Na_2CO_3 until final concentration of 50 mmol/L. The biotin probe was purified as the Tb-labelled one.

Coating of latex particles with streptavidin. Latex particles were coated with streptavidin as in Example 2 with antibody.

Hybridisation. PCR-amplified 170 bp part of Ph^1-chromosomal fraction from human K562 cell line (Turku University Central Hospital, Turku, Finland) served as the positive and distilled water as the negative control. Hybridisation was performed in 1.5-ml polypropylen centrifugal tubes presaturated with BSA. Coated latex particles (20 µL; about 100 million particles) were added. Positive sample and negative control were kept at 100 °C for 10 min and then cooled on an ice bath and they were centrifuged with a microcentrifuge for 1 min at 12,000 rpm. Fifty µL of a sample was applied onto latex particles and then 150 µL of assay buffer containing 2 ng of biotinylated probe and 2 ng Tb-labelled probe were added. The assay buffer contained 33.72 g of NaCl, 0.25 g NaN_3, 2.5 g BSA, 0.25 g bovine serum gammaglobulin and 0.05 mL of Tween 40 per litre of 25 mM Tris-HCl-buffer, pH 7.75. The reaction took place for 2 h at 50 °C. Then the particles were separated by a centrifuge and washed as in Example 5. Eventually the particles were suspended in 0.05 M $Na_2B_4O_7$ and were injected into a test tube (in a luminometer with a 545-nm interference filter) containing 10.0 mg of UV-irradiated $K_2S_2O_8$. Results are shown in Fig. 15.

Claims

1. A method, wherein an emission from a luminophore in a bioaffinity assay is obtained by an excitation of the luminophore with an energy released or formed by dissolution of the solid material, the energy being stored into the said material with techniques describable by general reactions:

$$\text{alkalihalides} \xrightarrow{\text{alkali metal vapour under high pressure and temperature}} F_{centres},$$

$$\text{alkalihalides} \xrightarrow{\text{halogen gas under high pressure and temperature}} V_{centres},$$

or with electrolytic coloration techniques according to general reactions:

$$\text{alkalihalides} \xrightarrow{\text{pointed cathode under high temperature}} F_{centres},$$

$$\text{alkalihalides} \xrightarrow{\text{pointed anode under high temperature}} V_{centres},$$

or with UV irradiation according to general reactions:

$$S_2O_8^{2-} \xrightarrow{h\nu > 4eV} 2\ SO_4^{\cdot -},$$

$$P_2O_8^{2-} \xrightarrow{h\nu > 4eV} 2\ PO_4^{\cdot 2-},$$

$$NO_3^- \xrightarrow{h\nu > 4.8\ eV} \text{peroxonitrite},$$

or achieved by dissolving a strongly reducing metal, like aluminium or magnesium, into an aqueous solution, thereby the method exploiting luminescent labels whose excitation is accomplished by the energy released or generated within the dissolution of the said solid materials into a solvent, and the said energy is converted to a measurable light emitted from the said luminescent label compounds, enabling the amount of the analyte to be quantified.

2. A method according to Claim 1, wherein luminescence from a label compound is generated in a solution with redox reaction mechanisms by the general reactions:

$$\text{solid} \xrightarrow{\text{solvent}} \text{dissolved solid} + \text{intermediates},$$

$$\text{intermediates} + \text{coreactant} \rightarrow \text{secondary intermediates produced by coreactant}$$

in which the intermediates generated during dissolution of the solid are either reducing species such as low-valent metal ions, hydrated electrons, hydrogen atoms; or oxidising species such as sulfate, hydroxyl or phosphate radicals, or halide or pseudohalide-containing radicals; and coreactant is a peroxosalt, an amine, oxalate or formiate, and in which the excitation of label occurs by succesive reactions according some of the reactions from the series:

$$A + \text{reducing intermediate} \rightarrow A_{red} + \text{intermediate product},$$

$$A + \text{oxidising intermediate} \rightarrow A_{ox} + \text{intermediate product},$$

$$A_{red} + \text{oxidising intermediate} \rightarrow A^* + \text{intermediate product},$$

$$A_{ox} + \text{reducing intermediate} \rightarrow A^* + \text{intermediate product},$$

$$A_{red} + A_{ox} \rightarrow A^* + A,$$

$$A^* \rightarrow A + h\nu \text{ (light)},$$

in which equations, A is an excitable label, A_{red} a one-electronically reduced form of the label, and A_{ox} a one-electronically oxidised form of the label, and reducing and oxidising

intermediate is one of the one of the dissolution-produced primary intermediates or secondary intermediates produced from coreactants; or by general reactions:

Luminophore + oxidising intermediates → luminophore intermediates,

luminophore intermediates → products + $h\nu$,

or the luminescence from label can be formed via energy transfer by general reactions:

$$e_{aq}^- + X\cdot \rightarrow (X^-)^*$$
$$(X^-)^* + A \rightarrow X^- + A^*$$
$$A^* \rightarrow A + h\nu \text{ (light)}$$

wherein A represents the excitable label and $X\cdot$ is an oxidising radical like sulfate, phosphate, or hydroxyl radical, or other radical, generated from halide or pseudo-halide ions by the said radicals.

3. A method according to Claim 1 and 2, wherein emitting light from a label compound enables to quantify the amount of an analyte with a lyoluminometer, or with another related equipment, by bringing a label into the measuring chamber of the said device, wherein the conditions defined in Claims 1 and 2 are generated, and the analyte is quantified by the luminescence from label monitored during a suitable period of time.

4. A method according to Claim 1 and 2, wherein the bioaffinity reaction is carried out on a surface of solid support, and wherein a label, labelled antibody, or labelled antigen; the label being a luminophore excitable by extrinsic lyoluminescence mechanisms of Claims 1 and 2, is released from the solid phase into solution after bioaffinity reaction and washing by disrupting the bonds between biomolecules, or by disrupting the bond(s) between label and the compound having been labelled with a change of one or more of the adjustable parameters of pH, temperature, UV-light, ultrasound, detergent, chaotropic agent, strongly reducing or oxidising conditions, organic solvent, or high ionic strength.

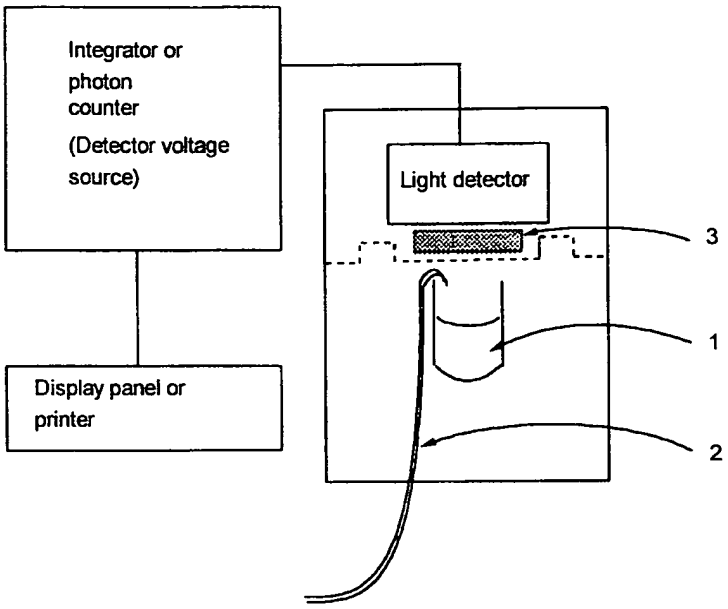
Integrator or
photon
counter
(Detector voltage
source)
Light detector
3
1
2
Display panel or
printer

Fig. 1.

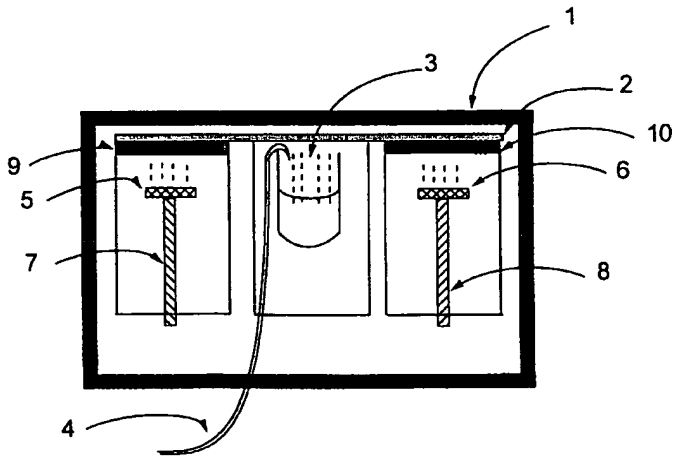
1
3
2
10
9
6
5
7
8
4

Fig. 2.

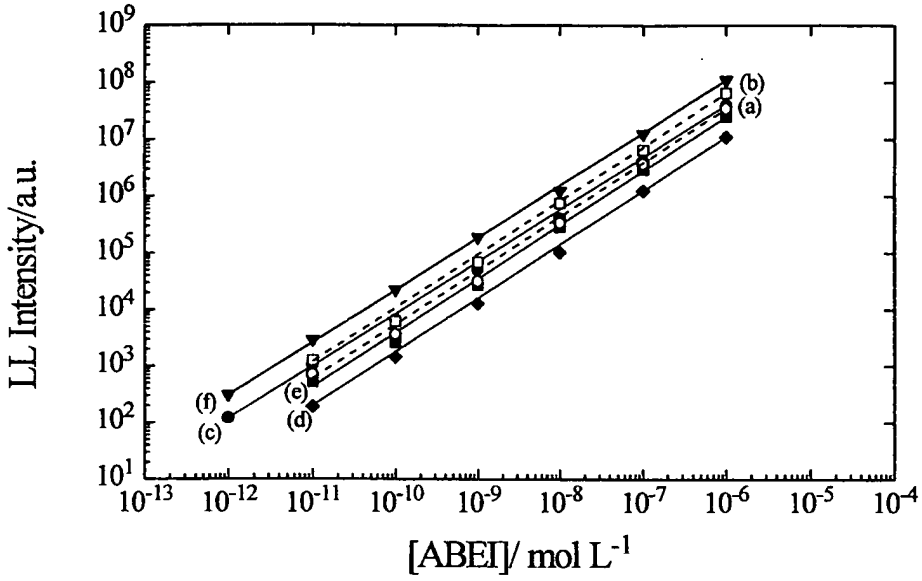
LL Intensity/a.u.
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
(b)
(a)
(f)
(e)
(c)
(d)
10^{-13} 10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4}
[ABEI]/ mol L^{-1}

Fig. 3.

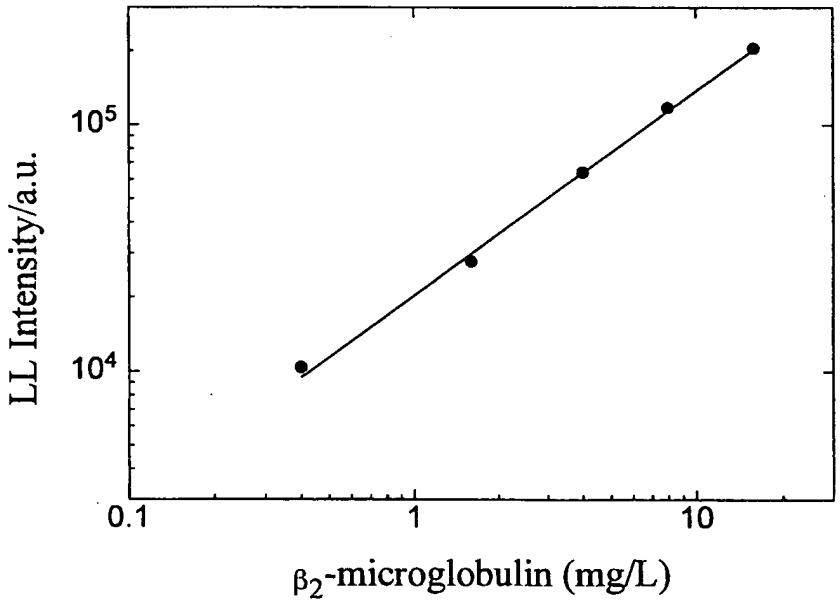
LL Intensity/a.u.
10^5
10^4
0.1
1
10
β_2-microglobulin (mg/L)

Fig. 4.

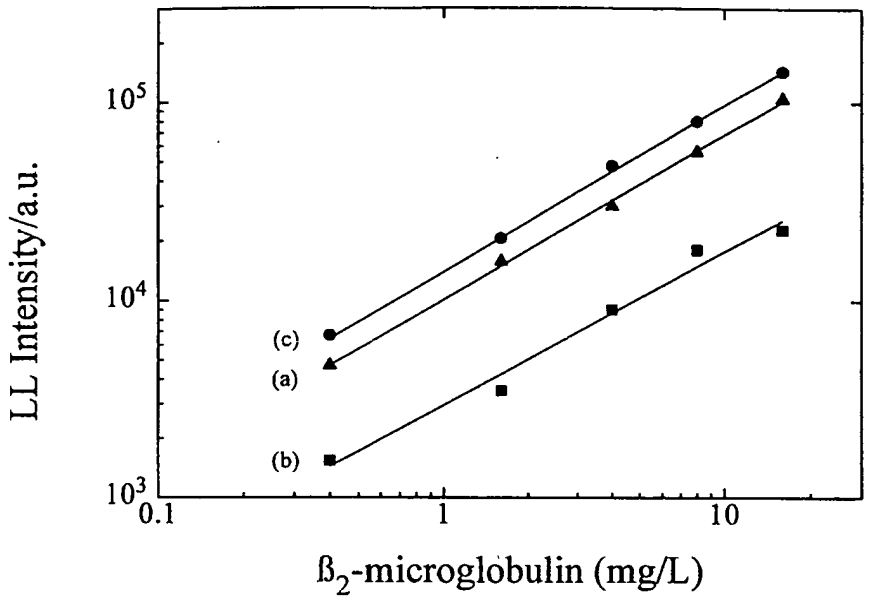
LL Intensity/a.u.
10^5
10^4
10^3
0.1
1
10
(c)
(a)
(b)
β_2-microglobulin (mg/L)

Fig. 5.

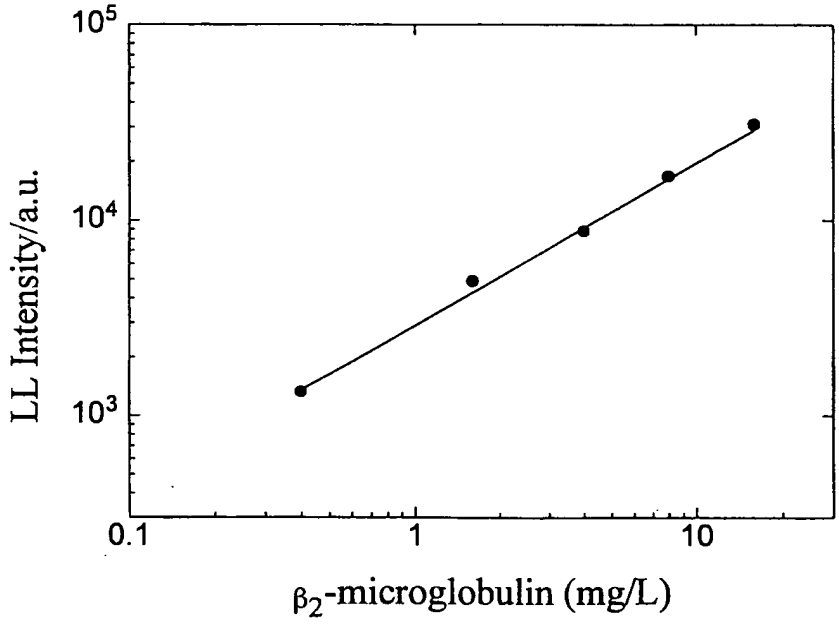
LL Intensity/a.u.
10^5
10^4
10^3
0.1
1
10
β_2-microglobulin (mg/L)

Fig. 6.

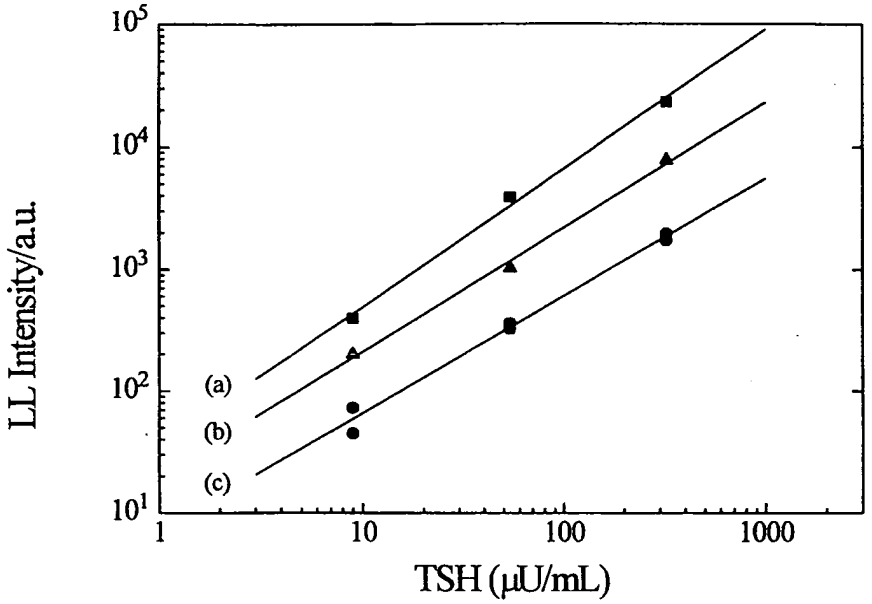
LL Intensity/a.u.
10^5
10^4
10^3
10^2
10^1
(a)
(b)
(c)
1
10
100
1000
TSH (μU/mL)

Fig. 7.

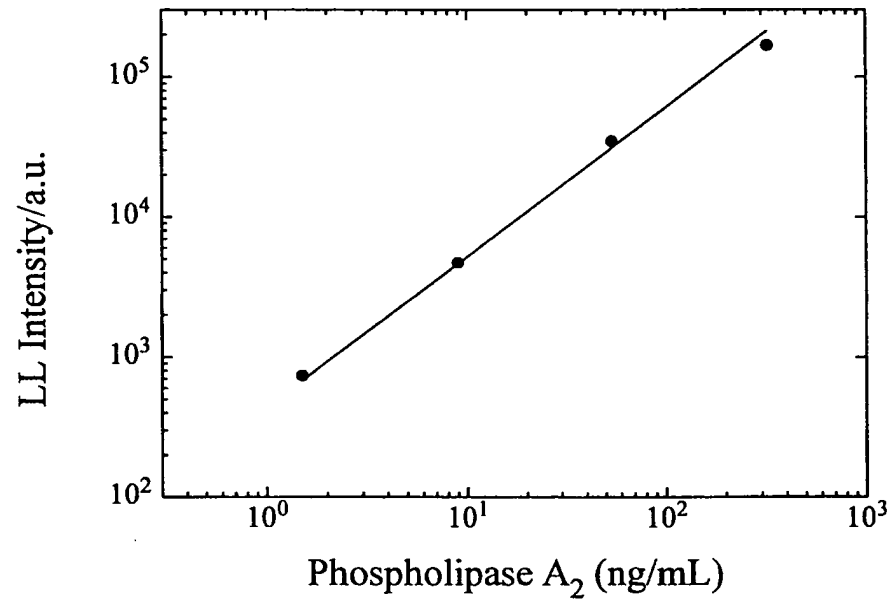
LL Intensity/a.u.
10^5
10^4
10^3
10^2
10^0
10^1
10^2
10^3
Phospholipase A_2 (ng/mL)

Fig. 8.

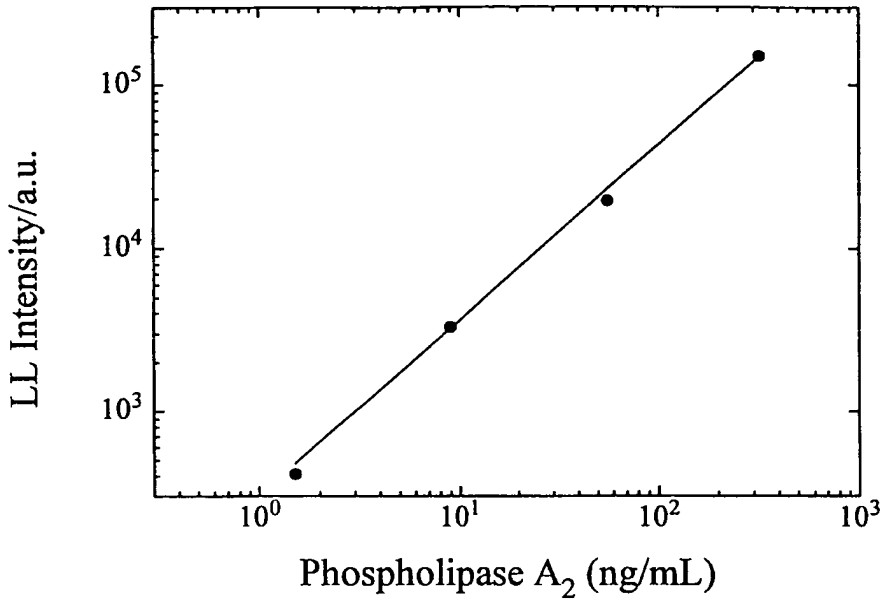
LL Intensity/a.u.
10^5
10^4
10^3
10^0
10^1
10^2
10^3
Phospholipase A_2 (ng/mL)

Fig. 9.

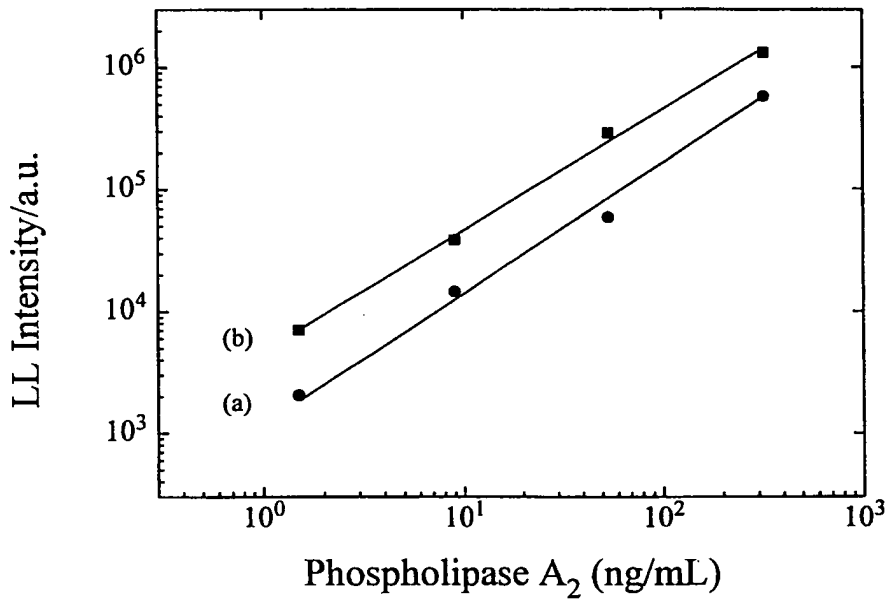
LL Intensity/a.u.
10^6
10^5
10^4
10^3
(b)
(a)
10^0
10^1
10^2
10^3
Phospholipase A_2 (ng/mL)

Fig. 10.

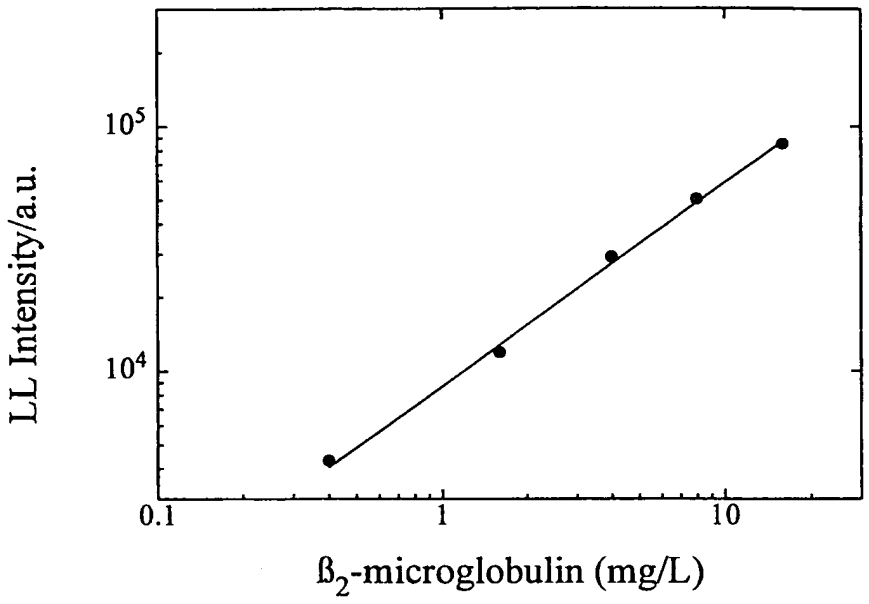
LL Intensity/a.u.
10^5
10^4
0.1
1
10
ß$_2$-microglobulin (mg/L)

Fig. 11.

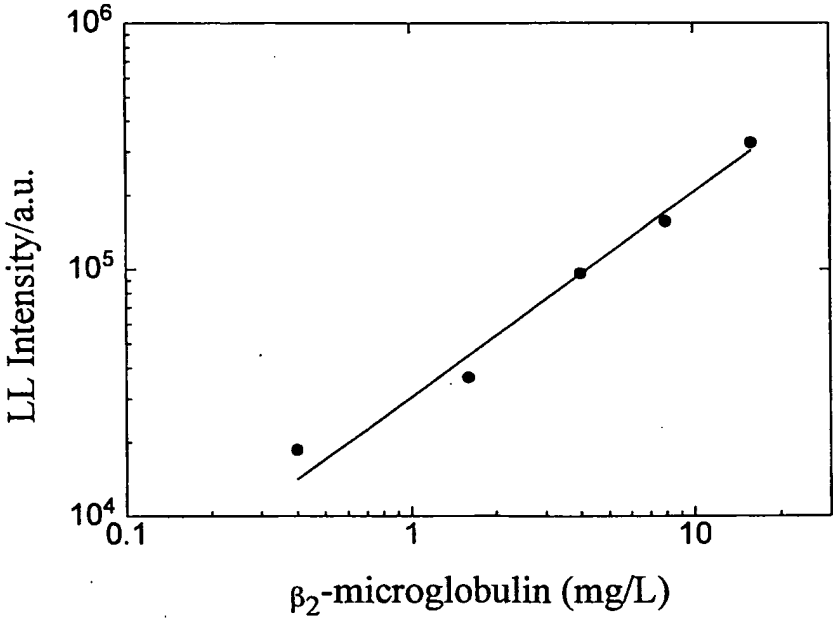
LL Intensity/a.u.
10^6
10^5
10^4
0.1
1
10
β$_2$-microglobulin (mg/L)

Fig. 12.

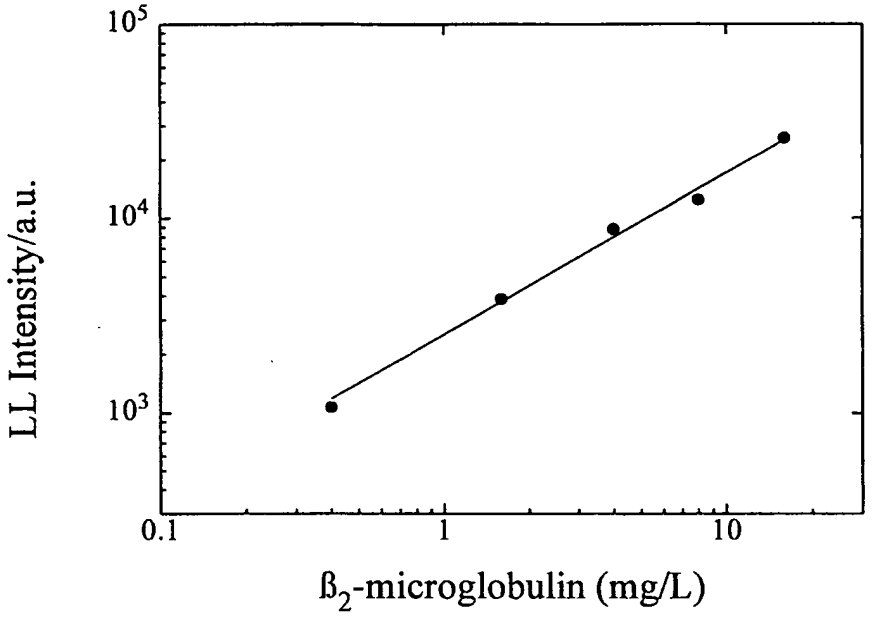
10^5
10^4
10^3
LL Intensity/a.u.
0.1
1
10
ß$_2$-microglobulin (mg/L)

Fig. 13.

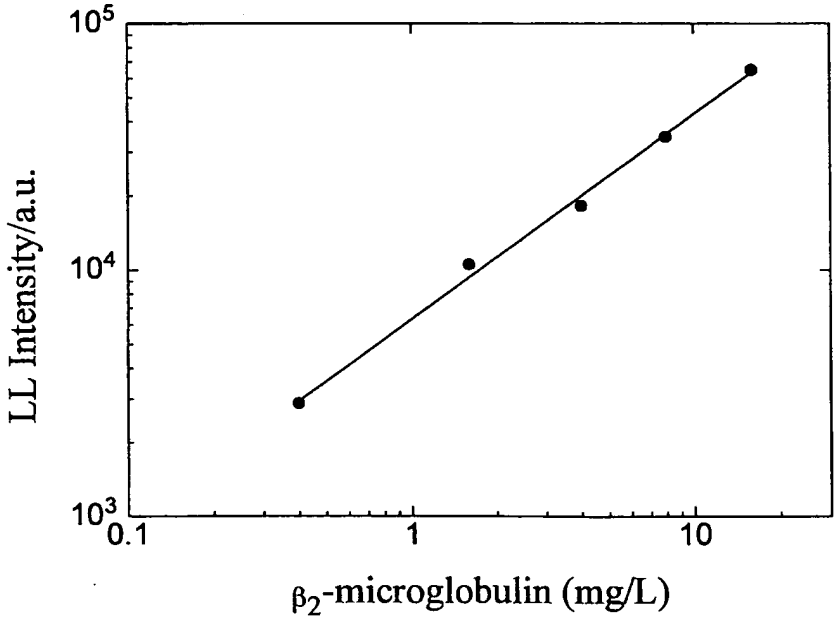
10^5
10^4
10^3
LL Intensity/a.u.
0.1
1
10
β$_2$-microglobulin (mg/L)

Fig. 14.

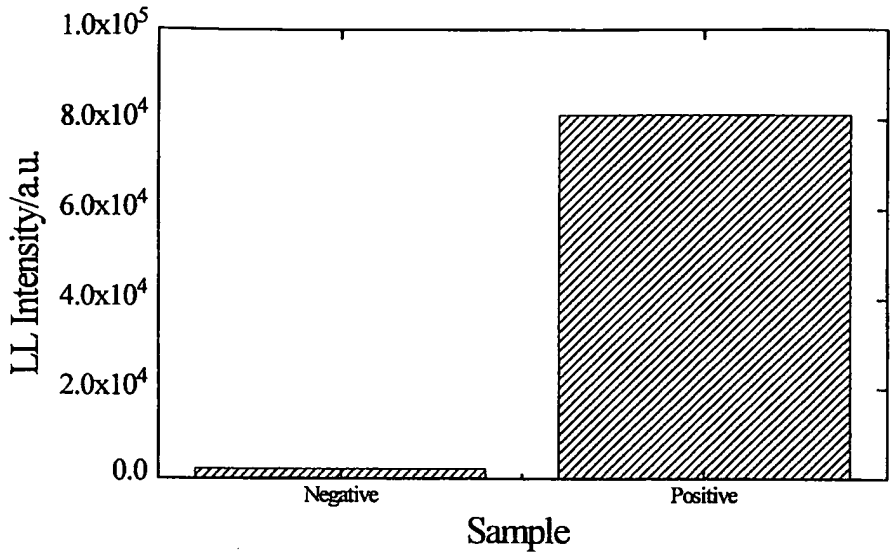
1.0x10^5
8.0x10^4
6.0x10^4
4.0x10^4
2.0x10^4
0.0
LL Intensity/a.u.
Negative
Positive
Sample

Fig. 15.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 98/00260

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: G01N 33/532, G01N 33/58
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Dialog Information Services, file 34, SciSearch, Dialog accession no. 06034803, Matachescu C et al: "Mechanism and analytical applicability of luminol specific extrinsic lyoluminescence of UV-irradiated potassium peroxodisulfate"; & ANALYTICAL CHEMISTRY, 1997, V69, N16 (AUG 15), P3385-3390 -- --------	1

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 June 1998	01 -07- 1998
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carl-Olof Gustafsson Telephone No. +46 8 782 25 00